

The Examiner has made Claims 1-20 subject to a restriction requirement under 35 U.S.C. 121. The Examiner has divided Claims 1-20 into eight groups, a summary of which follows:

- I. Claims 1, 8, 9, 12, 19 limited to subgenera G1 and G3;
- II. Claims 1-6, 8, 12-16, 19 limited to subgenera G1 and G4;
- III. Claims 1, 8, 9, 12, 19 limited to subgenera G2 and G3;
- IV. Claims 1-6, 8, 12-16, 19 limited to subgenera G2 and G4;
- V. Claims 17-18, drawn to a method of using the compounds of Group I;
- VI. Claims 17-18, drawn to a method of using the compounds of Group II;
- VII. Claims 17-18, drawn to a method of using the compounds of Group III;
and
- VIII. Claims 17-18 drawn to a method of using the compounds of Group IV.

Restriction is only proper if the restricted inventions are independent and patentably distinct (35 USC §121) and there is a *serious burden* placed on an Examiner if restriction is not required (MPEP 803). The burden is on an Examiner to provide reasons and/or examples to support any conclusions of patentable distinctness between the restricted inventions (MPEP 803). Applicants respectfully traverse the Restriction Requirement on the grounds that no adequate reasons and/or examples have been provided to support a conclusion of patentable distinctness between the restricted inventions and that no serious burden is placed on the Examiner if restriction is not required.

For the above reasons, Applicants submit that the restriction by the Examiner is improper and request that the restriction be withdrawn or reconsidered.

Respectfully submitted,



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UTILITY

The compounds of Formula (I) are expected to inhibit the activity of Hepatitis C Virus NS3 protease. The NS3 protease inhibition is demonstrated using assays for NS3 protease activity, for example, using the assay described below for assaying inhibitors of NS3 protease. The compounds of Formula (I) are expected to show activity against NS3 protease in cells, as demonstrated by the cellular assay described below. Thus, the compounds of Formula (I) are potentially useful in the cure and prevention of HCV infections.

Expression and Purification of NS3 Protease

The plasmid cf1SODp600, containing the complete coding region of HCV NS3 protease, genotype 1a, was obtained from ATCC (database accession: DNA Seq. Acc. M62321, originally deposited by Chiron Corporation). PCR primers were designed that allow amplification of the DNA fragment encoding the NS3 protease catalytic domain (amino acids 1 to 192) as well as its two N-terminal fusions, a 5 amino acid leader sequence MGAQH (SEQ. ID. NO.:1) (serving as a expression tag) and a 15 amino acid His tag MRGSHHHHHMGAQH (SEQ. ID. NO.:2). The NS3 protease constructs were cloned in the bacterial expression vector under the control of the T7 promoter and transformed in *E. coli* BL 21 (DE3) cells. Expression of the NS3 protease was obtained by addition of 1 mM IPTG and cells were growing for additional 3h at 25°C. The NS3 protease constructs have several fold difference in expression level, but exhibit the same level of solubility and enzyme specific activity. A typical 10 L fermentation yielded approximately 200 g of wet cell paste. The cell paste was stored at -80°C. The NS3 protease was purified based on published procedures (Steinkuhler C. et al. *Journal of Virology* 70, 6694-6700, 1996 and Steinkuhler C. et al. *Journal of Biological Chemistry* 271, 6367-6373, 1996.) with some modifications. Briefly, the cells were

5 resuspended in lysis buffer (10 mL/g) containing PBS buffer
(20 mM sodium phosphate, pH 7.4, 140 mM NaCl), 50%
glycerol, 10 mM DTT, 2% CHAPS and 1mM PMSF. Cell lysis was
performed with use of microfluidizer. After homogenizing,
DNase was added to a final concentration 70 U/mL and cell
10 lysate was incubated at 4°C for 20 min. After
centrifugation at 18,000 rpm for 30 min at 4°C supernatant
was applied on SP Sepharose column (Pharmacia), previously
equilibrated at a flow rate 3 mL/min in buffer A (PBS
buffer, 10% glycerol, 3 mM DTT). The column was extensively
15 washed with buffer A and the protease was eluted by
applying 25 column volumes of a linear 0.14 - 1.0 M NaCl
gradient. NS3 containing fractions were pooled and
concentrated on an Amicon stirred ultrafiltration cell
using a YM-10 membrane. The enzyme was further purified on
20 26/60 Superdex 75 column (Pharmacia), equilibrated in
buffer A. The sample was loaded at a flow rate 1 mL/min,
the column was then washed with a buffer A at a flow rate 2
mL/min. Finally, the NS3 protease containing fractions were
applied on Mono S 10/10 column (Pharmacia) equilibrated in
25 50 mM Tris.HCl buffer, pH 7.5, 10% glycerol and 1 mM DTT
and operating at flow rate 2 mL/min. Enzyme was eluted by
applying 20 column volumes of a linear 0.1 - 0.5 M NaCl
gradient. Based on SDS-PAGE analysis as well as HPLC
analysis and active site titration, the purity of the HCV
30 NS3 1a protease was greater than 95%. The enzyme was stored
at -70°C and diluted just prior to use.

Enzyme Assays

Concentrations of protease were determined in the absence
35 of NS4a by using the peptide ester substrate Ac-
DED(Edans)EEAbuψ[COO]ASK(Dabcy1)-NH₂ **(SEQ. ID. NO.:3)**
(Taliani et al. *Anal. Biochem.* 240, 60-67, 1996.) and the
inhibitor, H-Asp-Glu-Val-Val-Pro-boroAlg-OH **[and the**
inhibitor, H-Asp-Glu-Val-Val-Pro-boroAlg-OH] (SEQ. ID.
40 **NO.:5)** and by using tight binding reaction conditions
(Bieth, *Methods Enzymol.* 248, 59-85, 1995). Best data was

5 obtained for an enzyme level of 50 nM. Alternately,
protease (63 $\mu\text{g/mL}$) was allowed to react with 3 μM NS4a,
0.10 mM Ac-Glu-Glu-Ala-Cys-pNA (SEQ. ID. NO.:4), and
varying level of H-Asp-Glu-Val-Val-Pro-boroAla-OH (0-6 μM).
Concentrations of protease were determined from linear
10 plots of Activity vs.[inhibitor]. Molar concentrations of
proteases were determined from the x-intercept.

K_m values were determined measuring the rate of
hydrolysis of the ester substrate over a range of
concentrations from 5.0 to 100 μM in the presence of 3 μM
15 KKNS4a (KKGSVVIVGRIVLSGKPAIIPKK) (SEQ. ID. NO.:6). Assay
were run at 25°C, by incubating ~1 nM enzyme with NS4a for 5
min in 148 μl of buffer (50 mM Tri buffer, pH 7.0, 50%
glycerol, 2% Chaps, and 5.0 mM DTT. Substrate (2.0 μl) in
buffer was added and the reaction was allowed to proceed
20 for 15 min. Reactions were quenched by adding 3.0 μL of 10%
TFA, and the levels of hydrolysis were determined by HPLC.
Aliquots (50 μL) were injected on the HPLC and linear
gradients from 90% water, 10% acetonitrile and 0.10 % TFA
to 10% water, 90% acetonitrile and 0.10% TFA were run at a
25 flow rate of 1.0 mL/min over a period of 30 min. HPLCs were
run on a HP1090 using a Rainin 4.6 x 250 mm C18 column (cat
83-201-C) fluorescent detection using 350 and 500 nm as
excitation and emission wavelengths, respectively. Levels
of hydrolysis were determined by measuring the area of the
30 fluorescent peak at 5.3 min. 100% hydrolysis of a 5.0 μM
sample gave an area of 7.95 ± 0.38 fluorescence units.).
Kinetic constants were determined from the iterative fit of
the Michaelis equation to the data. Results are consistent
with data from Liveweaver Burk fits and data collected for
35 the 12.8.min peak measured at 520 nm.

Enzyme activity was also measured by measuring the
increase in fluorescence with time by exciting at 355 nm
and measuring emission at 495 nm using a Perkin Elmer LS 50

5 spectrometer. A substrate level of 5.0 μM was used for all
fluorogenic assays run on the spectrometer.

Inhibitor Evaluation In vitro

Inhibitor effectiveness was determined by measuring
10 enzyme activity both in the presence and absence of
inhibitor. Velocities were fit to the equation for
competitive inhibition for individual reactions of
inhibitors with the enzyme using

$$v_i / v_o = [K_m (1 + I/K_i) + S] / [K_m + S].$$

15 The ratio v_i / v_o is equal to the ratio of the
Michaelis equations for velocities measured in the presence
(v_i) and absence (v_o) of inhibitor. Values of v_i / v_o were
measured over a range of inhibitor concentrations with the
aid of an Excel™ Spreadsheet. Reported K_i values are the
20 average of 3-5 separate determinations. Under the
conditions of this assay, the IC_{50} and K_i s are comparable
measures of inhibitor effectiveness.

Using the methodology described above, compounds of
the present invention were found to exhibit a K_i of $\leq 60 \mu\text{M}$,
25 thereby confirming the utility of the compounds of the
present invention as effective NS3 protease inhibitors.
Preferred compounds of the present invention have K_i 's of
 $\leq 1 \mu\text{M}$. More preferred compounds of the present invention
have K_i 's of $\leq 100 \text{ nM}$. Most preferred compounds of the
30 present invention have K_i 's of $\leq 10 \text{ nM}$.

Inhibitor Evaluation in Cell Assay.

The following method was devised to assess inhibitory
action of test compounds on the HCV NS3 protease in
35 cultured cells. Because it is not possible to efficiently
infect cells with hepatitis C virus, an assay was developed
based on co-expression in transfected cell lines of two
plasmids, one is able to direct synthesis of the NS3
protease and the other to provide a polypeptide analogous
40 to a part of the HCV non-structural protein containing a
single known peptide sequence highly susceptible to

5 cleavage by the protease. When installed in cultured cells
by one of a variety of standard methods, the substrate
plasmid produces a stable polypeptide of approximately
50KD, but when the plasmid coding for the viral protease is
co-expressed, the enzymatic action of the protease
10 hydrolyzes the substrate at a unique sequence between a
cysteine and a serine pair, yielding products which can be
detected by antibody-based technology, eg, a western blot.
Quantitation of the amounts of precursor and products can
be done by scanning film auto-radiograms of the blots or
15 direct luminescence-based emissions from the blots in a
commercial scanning device. The general organization of the
two plasmids is provided in Scheme 6. The coding sequences
for the NS3 protease and the substrate were taken from
genotype 1a of HCV, but other genotypes, eg 2a, may be
20 substituted with similar results.

The DNA plasmids are introduced into cultured cells
using electroporation, liposomes or other means. Synthesis
of the protease and the substrate begin shortly after
introduction and may be detected within a few hours by
25 immunological means. Therefore, test compounds are added at
desired concentrations to the cells within a few minutes
after introducing the plasmids. The cells are then placed
in a standard CO₂ incubator at 37°C, in tissue culture
medium eg Dulbecco-modified MEM containing 10% bovine
30 serum. After 6-48 hours, the cells are collected by
physically scraping them from plastic dishes in which they
have been growing, centrifuging them and then lysing about
10⁶ of the concentrated cells in a minimal volume of
buffered detergent, eg 20 µl of 1% sodium dodecyl sulfate
35 in 0.10 M Tris-HCl, pH 6.5, containing 1% mercaptaethanol
and 7% glycerol. The samples are then loaded onto a
standard SDS polyacrylamide gel, the polypeptides separated
by electrophoresis, and the gel contents then
electroblotted onto nitrocellulose or other suitable paper
40 support, and the substrate and products detected by
decoration with specific antibodies.

5 Although this invention has been described with
respect to specific embodiments, the details of these
embodiments are not to be construed as limitations. Various
equivalents, changes and modifications may be made without
departing from the spirit and scope of this invention, and
10 it is understood that such equivalent embodiments are part
of this invention.

Preparation of H-Asp-Glu-Val-Val-Pro-boroAlg pinanediol
ester•trifluoroacetate (SEQ. ID. NO.:7).

15

Preparation of Boc-Asp(O^tBu)-Glu(O^tBu)-Val-Val-Pro-OH (SEQ.
ID. NO.:8). Boc-Val-Pro-OBzl was prepared by dissolving H-
Pro-OBzl (20 g, 83 mmol) in 50 mL of chloroform and adding
Boc-Val-OH (18.0 g, 83 mmol), HOBt (23.0g, 165 mmol), NMM (
20 9.0 mL, 83 mmol) and DCC (17.0 g, 83 mmol). The reaction
mixture was stirred overnight at room temperature. The
mixture was filtered and solvent was evaporated. Ethyl
acetate was added and insoluble material was removed by
filtration. The filtrate was washed with 0.2N HCl, 5%
25 NaHCO₃, and saturated aqueous NaCl. It was dried over
Na₂SO₄, filtered and evaporate to give a white solid (30 g,
75 mmol, 90%). ESI/MS calculated for C₂₂H₃₂N₂O₅ +H: 405.2.
Found 405.6.

30 Boc-Val-Val-Pro-OBzl was prepared by dissolving Boc-Val-
Pro-OBzl (14.0 g, 35.0 mmol) in 4N HCl in dioxane (20 mL)
and allowing the reaction to stir for 2h under an inert
atmosphere at room temperature. The reaction mixture was
concentrated by evaporation *in vacuo* and ether was added to
35 yield a precipitate. It was collected by filtration under
nitrogen. After drying *in vacuo* with P₂O₅, H-Val-Pro-OBzl
was obtained as a white solid (22.6 g, 30.3 mmol, 89%).
(ESI/MS calculated for C₁₇H₂₄N₂O₃ +H: 305.2. Found:
305.2.) H-Val-Pro-OBzl (9.2 g, 27 mmol) was dissolved in
40 50 mL of CH₂Cl₂ and Boc-Val-OH (7.3 g, 27 mmol), HOBt (7.3
g, 54 mmol), NMM (3.0 mL, 27 mmol) and DCC (5.6 g, 27 mmol)

5 were added. The reaction mixture stirred overnight at room temperature. The mixture was filtered and the filtrate was evaporated. The residue was dissolved in ethyl acetate and the solution was re-filtered. The filtrate was washed with 0.2N HCl, 5% NaHCO₃, and saturated aqueous NaCl. It was
10 dried over Na₂SO₄, filtered and evaporated to give a yellow oil (10.6 g, 21.1 mmol, 78%). ESI/MS calculated for C₂₇H₄₁N₃O₆ + Na: 526.3 Found: 526.4.

Z-Glu(O^tBu)-Val-Val-Pro-OBzl (SEQ. ID. NO.:9) was also
15 prepared by DCC coupling. H-Val-Val-Pro-OBzl·hydrochloride was obtained in a 100% yield by treating the corresponding Boc compound with anhydrous HCl using the procedure described for H-Val-Pro-OBzl (ESI/MS calculated for C₂₂H₃₃N₃O₄ + H: 404.2. Found 404.3.). The amine
20 hydrochloride (7.40 g, 16.8 mmol) was dissolved in 185 mL DMF and 25 mL THF. Z-Glu(O^tBu)-OH (5.60 g, 16.8 mmol), HOBT (4.60 g, 33.6 mmol), NMM (1.85 mL, 16.8 mmol) and DCC (3.5 g, 16.8 mmol) were added. The reaction was run and the product was isolated by the procedure described for
25 Boc-Val-Val-Pro-OBzl. The tetrapeptide was obtained as a white foam (12.0 g, 16.1 mmol, 96%). ESI/MS calculated for C₃₉H₅₄N₄O₉ + Na: 745.4. Found: 745.4.

H-Glu(O^tBu)-Val-Val-Pro-OH (SEQ. ID. NO.:10) was prepared
30 by dissolving Z-Glu(O^tBu)-Val-Val-Pro-OBzl (2.90 g, 3.89 mmol) in 100 mL methanol containing 1% acetic acid. Pearlman's catalyst, Pd(OH)₂, (100mg) was added and the flask was placed on the Parr hydrogenation apparatus with an initial H₂ pressure of 34 psi. After three hours, the
35 catalyst was removed by filtration through a celite pad and the filtrate was evaporated in vacuo to yield a yellow oil (1.30 g, 2.61 mmol, 67%). ESI/MS calculated for C₂₄H₄₂N₄O₇ +H: 499.3 Found: 499.4.

40 Boc-Asp(O^tBu)-Glu(O^tBu)-Val-Val-Pro-OH was prepared by active ester coupling. Boc-Asp(O^tBu)-N-hydroxysuccinimide

5 ester was prepared by coupling Boc-Asp(O^tBu)-OH (3.00 g, 10.4 mmol) to N-hydroxysuccinimide (1.19 g, 10.4 mmol) in 50 mL of ethylene glycol dimethyl ether. The reaction flask was placed in an ice bath at 0°C and DCC was added. The reaction mixture was slowly allowed to warm to room
10 temperature and to stir overnight. The mixture was filtered and the filtrate was evaporated *in vacuo*. The residue was dissolved in ethyl acetate and re-filtered. The filtrate was evaporated give a white solid. Recrystallized from ethyl acetate: hexane gave the
15 activated ester (3.38 g, 8.80 mmol, 84%). (ESI/MS calculated for C₁₇H₂₆N₂O₈ + H: 387.2. Found: 387.4.) H-Glu(O^tBu)-Val-Val-Pro-OH (5.40 g, 10.8 mmol) was dissolved in 100 mL of water. Sodium bicarbonate (0.92 g, 11.0 mmol) was added followed by triethylamine (2.30 mL, 16.5 mmol).
20 The N-hydroxysuccinimide ester (3.84 g, 10.0 mmol) was dissolved in 100 mL dioxane and was added to the H-Glu(O^tBu)-Val-Val-Pro-OH solution. The mixture stirred overnight at room temperature. Dioxane was removed *in vacuo* and 1.0 M HCl was added to give pH ~ 1. The product
25 was extracted into ethyl acetate. The ethyl acetate solution was washed with 0.2 N HCl, dried over sodium sulfate, filtered, and evaporated to yield a yellow oil (7.7 g, 10.0 mmol, 100%). ESI/MS calculated for C₃₇H₆₃N₅O₁₂ + Na: 792.4. Found: 792.4.
30
Boc-Asp(O^tBu)-Glu(O^tBu)-Val-Val-Pro-boroAlg-pinanediol (SEQ. ID. NO.:11) was prepared by coupling the protected pentapeptide to H-boroAlg-pinanediol. Boc-Asp(O^tBu)-Glu(O^tBu)-Val-Val-Pro-OH (1.8 g, 2.3 mmol) was dissolved 10
35 mL THF and was cooled to -20°C. Isobutyl chloroformate (0.30 mL, 2.3 mmol) and NMM (0.25 mL, 2.3 mmol) were added. After 5 minutes, this mixture was added to H-boroAlg-pinanediol (0.67 g, 2.3 mmol) dissolved in THF (8 mL) at -20°C. Cold THF (~5 mL) was used to aid in the transfer.
40 Triethylamine (0.32 mL, 2.3 mmol) was added and the reaction mixture was allowed to come to room temperature

5 and to stir overnight. The mixture was filtered and
solvent was removed by evaporation. The residue was
dissolved in ethyl acetate, washed with 0.2 N HCl, 5%
NaHCO₃, and saturated NaCl. The organic phase was dried
with Na₂SO₄, filtered, and evaporated to yield a yellow oil.
10 Half of the crude product (1.5 g) was purified in 250 mg
lots by HPLC using a 4 cm x 30 cm Rainin C-18 reverse phase
column. A gradient from 60: 40 acetonitrile: water to 100%
acetonitrile was run over a period of 28 minutes at a flow
rate of 40 mL/min. The fractions containing the desired
15 product were pooled and lyophilized to yield a white solid
(46 mg). ¹H-NMR (CD₃OD) δ 0.9-1.0 (m, 15H), 1.28 (s, 3H),
1.3 (s, 3H), 1.44 (3s, 27H), 1.6-2.8 (20H), 3.7(m, 1H),
3.9(m, 1H), 4.1-4.7 (7H), 5.05(m, 2H), 5.9(m, 1H). High
res (ESI/MS) calculated for C₅₁H₈₆N₆O₁₃B₁ +H: 1001.635.
20 Found 1001.633.

Preparation of H-Asp-Glu-Val-Val-Pro-boroAlg-pinanediol
ester•trifluoroacetate: The hexapeptide analog, Boc-
Asp(O^tBu)-Glu(O^tBu)-Val-Val-Pro-boroAlg-pinanediol, (22.5
25 mg, 0.023 mmol) was treated with 2 mL of TFA: CH₂Cl₂ (1: 1)
for 2 h. The material was concentrated *in vacuo* and
purified by HPLC using C-18 Vydac reverse phase (2.2 x 25
cm) column with a gradient starting at 60:40
acetonitrile/water with 0.1%TFA going to 95:5 over 25
30 minutes with a flow rate of 8 mL/min. The product eluted
at 80% acetonitrile. The fractions were evaporated and
dried under high vacuum to give 8.9 mg (49%) of the desired
product as white amorphous solid. ¹H-NMR (CD₃OD) δ 5.82
(m, 1H), 5.02 (m, 2H), 4.58(m, 1H), 4.42 (m, 3H), 4.18 (m,
35 4H), 3.90 (m, 1H), 3.62 (m, 1H), 3.01 (dd, 1H), 2.78 (m,
1H), 2.62 (m, 1H), 2.41-1.78 (m, 17H), 1.31 (s, 3H), 1.28
(s, 3H), 1.10 - 0.82 (m, 15H). ESI/MS calculated for
C₃₈H₆₂N₆O₁₁B +H: 789.2. Found: 789.2.